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DOI:

[10.1016/j.dental.2015.11.016](https://doi.org/10.1016/j.dental.2015.11.016)

Document Version

Publisher's PDF, also known as Version of record

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Citation for published version (APA):

Austin, R., Giusca, C. L., Macaulay, G., Moazzez, R., & Bartlett, D. W. (2016). Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralization and salivary remineralization in vitro. *Dental Materials*, 32(2), 278-284.
<https://doi.org/10.1016/j.dental.2015.11.016>

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Manuscript Number: DEMA-D-15-00144R1

Title: Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralisation and salivary remineralisation in vitro

Article Type: Full Length Article

Keywords: Erosion; surface texture; remineralisation; enamel; imaging; microhardness

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Order of Authors: Rupert Sloan Austin; Claudiu Giusca; Gavin Macaulay; Rebecca Moazzez; David Bartlett

Abstract: Objectives: This paper investigates the application of confocal laser scanning microscopy to determine the effect of acid-mediated erosive enamel wear on the micro-texture of polished human enamel in vitro. Methods: Twenty polished enamel samples were prepared and subjected to a citric acid erosion and pooled human saliva remineralisation model. Enamel surface microhardness was measured using a Knoop hardness tester, which confirmed that an early enamel erosion lesion was formed which was then subsequently completely remineralised. A confocal laser scanning microscopy was used to capture high-resolution images of the enamel surfaces undergoing demineralisation and remineralisation. Area-scale analysis was used to identify the optimal feature size following which the surface texture was determined using the 3D (areal) texture parameter Sa. Results: The Sa successfully characterised the enamel erosion and remineralisation for the polished enamel samples ($P < 0.001$). Significance: Areal surface texture characterisation of the surface events occurring during enamel demineralisation and remineralisation requires optical imaging instrumentation with lateral resolution $< 2.5 \mu\text{m}$, applied in combination with appropriate filtering in order to remove unwanted waviness and roughness. These techniques will facilitate the development of novel methods for measuring early enamel erosion lesions in natural enamel surfaces in vivo.

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Professor David Watts

Dental Materials Journal

Tuesday, October 06, 2015

Dear Professor David Watts

We wish to re-submit a revised manuscript entitled "Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralisation and salivary remineralisation in vitro" for consideration by Dental Materials.

We thank you and the reviewers for their comments which we have addressed as outlined below. We hope this addresses the reviewer points and that the readers of Dental Materials will be interested in the study's findings.

Reviewers' comments:

Reviewer #1:

- 1) Was there ethical approval for the use of human tissue in this study?
 - a. There was ethical approval for the use of Human Tissue in this study as stated in lines 30-32 '*Research ethics approval for use of Human Tissue in this study had been granted (REC number 09/H0808/109)*'
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 - b. Were the specimens polished on a grinding/polishing machine?
 - c. Was it done by hand or using a specimen holder?
 - d. Were the specimens polished for the same amount of time down to the same depth?
 - e. Results from our lab show that all of these factors can affect the initial roughness of enamel specimens, with in particular, differences in depth that the enamel is polished before erosion particularly affected results.

- Samples were ground and polished (Buehler Metaserv 3000 variable speed grinder-polisher and Vector™ LC power head) with Federation of European Producers of Abrasives (FEPA) standard silicon carbide sandpaper. Custom-made jigs were made from bis-acrylic composite and cold cure acrylic resin to fit the power head and hold the samples in place. A force of 10 g was applied to the centre of the sample and a speed of 300 rpm applied. Starting at 80 grit, for approximately 5 seconds, this produced an initial flat area on the enamel. At this stage, the samples were individually visually inspected after drying the surface with a tissue, and checking that an area of enamel (~1 x 2 mm) had been exposed. If there was not any exposed enamel the sample was then ground again for 5 more seconds and re-checked, until there was exposed enamel. After which the samples were then cycled through 180 (10 seconds), 600 (25 seconds), 1200 (30 seconds), 2400 (35 seconds) and 4000 (45 seconds) grits to produce a flat, highly polished enamel surface. Samples were ground/polished in batches, with the silicon carbide disks replaced every 16 samples. When the samples were not being polished they were stored in deionised water baths.
- To assess the amount of enamel removed, a digital calliper, Duratool D00325, was used to measure the height of several samples before and after the polishing procedure.
- After polishing, the samples were visually inspected for cracks and several were randomly chosen to assess the flatness tolerance by profilometry. To assess the flatness tolerance, the average curvature over the area of the polished enamel from 5 profile readings, from the lowest point to the highest, was not greater than 0.4 μm . The slope was corrected for before analysis via levelling of the surface by removing the plane of best fit using the least squares method.
- Results from our lab show that this very standardised process produces a very homogenous baseline S_a roughness, especially due to the use of the customised jigs which secure the samples in place during polishing.
- We agree that the influence of the depth of polishing can affect the resulting prism orientation and therefore the feature size which can in turn influence the filtering that is required ensuring that the most relevant data is selected. Mention of this has been added to the discussion on Page 9 paragraph 1.

3) Who makes Gpower software?

- a. GPower is statistical freeware which can be freely downloaded from the web, so as such it doesn't have a citable maker. I have cited the use of the software which describes how it is employed as described elsewhere.

4) How was the flatness tolerance of 0.4 μm decided up for the specimens and how was it measured?

- a. After polishing, the samples were visually inspected to rule out any cracks or defects and the flatness tolerance of the samples was then assessed using profilometry (White light confocal sensor on a XYRIS 2000, Taicaan Technologies, UK). To assess the flatness tolerance, the average curvature over the area of the polished enamel from 5 profile readings, from the lowest point to the highest, was calculated.
 - b. A value of 0.4 μm was chosen as in previous experiments this has been shown to be the lowest achievable tolerance in our laboratory using the procedures described above. We could therefore be confident that we had prepared samples with appropriate baseline textural characteristics.
- 5) Was the pH of the distilled water measured prior to specimen storage? The pH of distilled water can be anything from 7.4 down to 5 depending on how it has been stored. Clearly, any pH below 5.5 can cause erosion of the enamel so it is important that this was established at all points in the study otherwise the rinsing steps, for instance, may have caused increased erosion.
 - a. The distilled water was pH 6.8 and therefore should not have influenced the erosion model unduly. For example Barbour et al. showed that rinsing enamel with distilled water between immersion in distilled water produced no measurable softening with nanoindentation (Michele E. Barbour et al. 2003). We have added the pH of the distilled water to the text in order to clarify this.
- 6) In terms of the pooled saliva:
 - a. From how many people was saliva obtained?
 - b. How was the saliva mixed and stored after it was obtained?
 - c. Was the saliva stimulated prior to collection or unstimulated?
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 - Paraffin-stimulated whole mouth saliva samples were collected from 30 healthy volunteers, following previously published protocols [1]. The collected saliva was ice-chilled and pooled immediately after collection at -80°C for long-term storage. Prior to use, the frozen natural saliva was defrosted in ice time at room temperature $22 \pm 1^{\circ}\text{C}$. The calcium content was measured using inductively coupled plasma mass spectroscopy (ICP-MS) as described in Carpenter et al 2014.
 - We have included these details into the methods section
- 7) Knoop hardness tests
 - a. It is very annoying to see "gold standard microhardness test" and "gold standard microhardness data" all the time. Firstly, microhardness data really isn't a "gold standard" anyway; this is an overused term in dental research and has now become a cliché. Ok, if the authors want to describe it as a "gold standard method" that is OK once, but every time is ridiculous. I suggest that most instances of "gold standard" are deleted in all the text.

- We apologise for this overemphasis on this point and have deleted all but one reference to this in the text
- b. What are the details of the instrument used to measure the Knoop hardness?
- c. How was the accuracy of the tester assessed?
- d. How did the authors ensure that indents were sufficiently far apart from each other that there was no interaction between indents next to each other, either taken at the same time period or at later erosion or remineralisation time points?
 - The Knoop microhardness tester was a Duramin-5 Hardness Tester (Struers Inc., Rotherham, UK)
 - Each indentation made 100 μm apart, to ensure that there were no interaction between indents next to each other. Each time the sample was repositioned, the computer video interface was used to examine the surface to ensure that no indents were placed closer than 100 μm from the adjacent indents. As this erosion model was an initial erosion model only with no bulk surface loss this aided the localisation and avoidance of previous indents.
 - The accuracy of the tester was measured using a 600 KHN calibrated transfer standard block (Staatliches Materialprüfungsamt Nordrhein-Westfalen, Dortmund, Germany).
 - These details have been added into the text of the manuscript

8) In terms of the filters applied to the confocal data:

- a. The authors describe the steps they used in the paper but they give no indication of how they assessed that these filters were the most appropriate. Did they apply other filters, not described in the paper, which did not improve the data? Or were the ones applied the only ones applied?
 - The selection of the filters was carried out using an iterative process within Mountains Map whereby representative sample images were taken from each erosion/demineralisation stage was chosen and the image analysis workflow performed within MountainsMap® in order to display the 3D data at each of the time points (baseline, during erosion and during remineralisation) in order to select the filters which produced the clearest visual highlighting of the feature of interest in the present study (i.e. the enamel prism structure). For each sample the filtering operators were then recalled and varying high-pass and low-pass filters were then employed. As a starting point firstly the data from the area scale analysis was used, secondly, the estimated noise floor according to the specific operating parameters of the CLSM and thirdly a features bases analysis algorithm was used to measure the diameter of the enamel prisms of the eroded samples in this study. These three sources of information were then used to determine the most likely low-pass filter and high-pass filters that would be used to remove the noise and the waviness thus leaving just the relevant surface texture data of the features of interest. As each varying filter was applied to the representative enamel samples at the differing time points, a real-time pseudo-colour visual image was automatically modified within

MountainsMap® which allowed the author to visually confirm whether the changes in the filters produced resulting data in which the prisms appeared as clearly as possible, especially after 5 minutes of erosion.

- A summary of this process has been included in the text
- b. In the results, the authors discuss the correlation between microhardness and scale of the relative area scale for erosion and remineralisation. In the erosion results, they show that the correlation reduces on areas greater than $20\ \mu\text{m}^2$. They then say that this suggests the $5\ \mu\text{m}^2$ is the correct scale for analysis; how do they arrive at $5\ \mu\text{m}^2$? This is not clear.
- Thank you for pointing this out – this was indeed a typo and should have referred to a minimum lateral resolution of approximately $2.5\ \mu\text{m}$ (for example a laser spot size on a profilometer or the x/y resolution of an optical instrument being well below this).
 - This is calculated according to the area-scale correlation data shown in Table 2 which shows that the correlation between the microhardness and the relative area-scale during demineralisation decreases dramatically (R^2 less than 0.8) for area scales greater than $20\ \mu\text{m}^2$. From this information, the authors calculated the minimal lateral resolution that is required to resolve features with an area less than $20\ \mu\text{m}^2$. Therefore, if the feature was circular the radius of a $20\ \mu\text{m}^2$ circle is approximately $2.5\ \mu\text{m}$ and this is that value that should be quoted.

If the authors address these points then I can see that the readers of Dental Materials will be interested in the study's findings.

Reviewer #2: Overall an interesting paper which adds value to the scientific research in this area. It self-identifies as a rangefinding experiment, to identify the optimal scale at which to carry out research into changes of surface texture during acid demin and remin. It achieves this aim, though it does raise some questions, which point to areas of further research.

Questions for the authors to resolve/comment on prior to publication.

- 1) Discussion, Page7: Why is $5\ \mu\text{m}$ recommended as the optimal resolution? The sentence before states there was a good correlation across the range of 0.1 to $20\ \mu\text{m}$. Therefore surely any measurements within this range would be equally valid? My reading of figures 2 and 3 is that this single experiment suggests anywhere within this range would work equally well, though further work may pinpoint the ideal scale range to be narrower. I appreciate that $5\ \mu\text{m}$ is near the middle of this range and probably well suited to this instrument. If those are the reasons for specifying $5\ \mu\text{m}$, rather than an optimal range, please make this clear. I believe this would be a valid conclusion for this instrument.
 - Following on from the previous responses above, we have presented the conclusion as a lateral resolution range from 50 nm to $2.5\ \mu\text{m}$, as explained earlier, which should address the concern raised above.

- 2) Fig 5 neatly shows the reduction in Sa as the surface roughness decreases during remineralisation. Questions on this graph:
- The delay in reduction of Sa begins somewhere between 1hr and 6 hrs into remineralisation. The author suggests pellicle formation may have played a role in no change in Sa seen up to 1hr. If this is true, could pellicle formation have confounded all remineralisation measures? Does this optical method measure the surface through the pellicle layer (in which case, pellicle would not affect the results, including the 1hr result) or does pellicle interfere (in which case all remin data should be considered with caution)? More discussion of this is recommended. I agree with the author's suggestion that future research should consider measurement of pellicle, or ultrasonic removal of pellicle. Or alternatively, use an artificial remin solution to remove the effects of pellicle completely, or test artificial remin solution vs natural saliva to test the pellicle hypothesis.
 - This is indeed the case that pellicle formation may have confounded all remineralisation measurements. The optical method measures in reflection mode and therefore the pellicle may have interfered with these measurements and the remineralisation data should indeed be treated with caution.
 - This has been emphasised in the text and more discussion has been made regarding possible future options to remedy this limitation of the present study
 - The graph suggests the reduction of Sa is still in steep decline at 12hrs. Is this decline expected to continue until baseline Sa value is reached? Is it expected to go beyond baseline Sa value? Discussion of this, or suggestion of further research.
 - Indeed, this may be a further issue related to the pellicle formation covering the enamel and preventing a true picture of the enamel texture.
 - Further discussion has been made in the manuscript
- 3) Fig 4 questions/comments.
- Fig 4A. no scale
 - Scales added
 - Fig 4B. Texture maps have widely different z scale, which has the potential to be misleading. For example, the 12 hr remin image looks much more textured than the 5 min erosion image. But this is an artefact, due to the much larger z range 5 min erosion image. I suggest a fixed z scale is employed for these 3 images, which will visually back up the numerical measurements. If not possible, I suggest the different z scales are called out in the figure description.
 - The different z-scales have been called out in the figure description
- 4) There is very little discussion of where else surface roughness has been measured after acid erosion and how changes seen in this pilot study compare. This may be quite a different instrument to those used elsewhere, but some discussion is warranted.
- More discussion has been added to the penultimate paragraph of the discussion section regarding alternative studies in this area and possibilities for future research

- 5) The author's bibliography contains a number of references outside the field of dentistry. This is to be commended. Roughness measurement is used extensively in other industries, and useful knowledge and method application can be gained from a wider literature search.
- 6) Note one typo: Page 2, materials and methods, paragraph 2. I believe 'citric acid power' should be 'citric acid powder'

- Corrected with thanks.

1. Carpenter, G., et al., Composition of enamel pellicle from dental erosion patients. Caries research, 2014. 48(5): p. 361-7.

Thank you for your consideration of this revised manuscript.

Sincerely,

A handwritten signature in black ink, appearing to read 'Rupert Austin'.

Dr Rupert Austin BDS (Hons) PhD MJDF RCS Eng FAcadMed FHEA

Date: May 07, 2015

To: "Rupert Sloan Austin" rupert.s.austin@kcl.ac.uk

From: "Dental Materials" dentistry.dentmatj@manchester.ac.uk

Subject: Your Submission DEMA-D-15-00144

Ms. Ref. No.: DEMA-D-15-00144

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 - b. Fig 4B. Texture maps have widely different z scale, which has the potential to be misleading. For example, the 12 hr remin image looks much more textured than the 5 min erosion image. But this is an artefact, due to the much larger z range 5 min erosion image. I suggest a fixed z scale is employed for these 3 images, which will visually back up the numerical measurements. If not possible, I suggest the different z scales are called out in the figure description.
 - The different z-scales have been called out in the figure description
- 4) There is very little discussion of where else surface roughness has been measured after acid erosion and how changes seen in this pilot study compare. This may be quite a different instrument to those used elsewhere, but some discussion is warranted.
- a. More discussion has been added to the penultimate paragraph of the discussion section regarding alternative studies in this area and possibilities for future research
- 5) The author's bibliography contains a number of references outside the field of dentistry. This is to be commended. Roughness measurement is used extensively in other industries, and useful knowledge and method application can be gained from a wider literature search.
- 6) Note one typo: Page 2, materials and methods, paragraph 2. I believe 'citric acid power' should be 'citric acid powder'
- Corrected with thanks.
1. Carpenter, G., et al., Composition of enamel pellicle from dental erosion patients. Caries research, 2014. 48(5): p. 361-7.

Title page

Title: Confocal laser scanning microscopy and area-scale analysis used to quantify enamel surface textural changes from citric acid demineralisation and salivary remineralisation *in vitro*

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Keywords

Erosion; surface texture; remineralisation; enamel; imaging; microhardness

Declaration of interests:

'Conflicts of interest: none'

Declaration of funding:

This project was supported by an award from the Academy of Medical Sciences Starter Grant for Clinical Lecturers Scheme which is funded by the Academy of Medical Sciences, the Wellcome Trust, the British Heart Foundation and Arthritis Research UK.

Thank you to Professor Christopher Brown for providing access to Sfrax software.

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Role of the funding source

The funding source(s) had no involvement in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

1.1 Introduction

Currently, no reliable technique exists for the quantification of early enamel erosion *in vivo*. *In vitro*, microhardness is considered the gold standard for characterising the early enamel erosion lesion [1, 2], however as this technique cannot be used *in vivo* there has recently been increased interest in characterising textural changes that occur during early erosive demineralisation and remineralisation [3-6]. In order to reliably quantify dynamic changes occurring in the enamel surface during acid mediated erosion, appropriate surface texture instrumentation and software would need to be carefully chosen in order to image the micro-scale features of exposed enamel rods or prisms [6-8].

Recent investigations into early erosive wear have suggested that enamel surface texture characterisation could be a suitable target for therapeutic oral care products [5, 9]. Moreover, in order to apply these analytical techniques *in vivo*, data on the optimal scale at which to measure these surface events are required in order to guide the choice of instrument selection, especially in terms of minimum lateral resolution required. To date, scale dependant relative area analysis used in anthropological micro-wear measurement [10] have yet to be applied to determine how to optimally employ 3D 'areal' surface texture analysis [11, 12] in order to characterise the surface events occurring in human enamel during acid initiated erosive wear and salivary mediated remineralisation [2, 4].

The aim of this study was therefore to determine the optimal scale at which enamel surface textural changes from citric acid demineralisation and salivary remineralisation can be observed *in vitro*, using confocal laser scanning microscopy. The objective was to utilise this clinically relevant enamel erosion model to determine the optimal surface texture measurement workflow to best characterise the enamel surface events occurring during erosion *in vitro*. The null hypothesis was that surface texture analysis with a confocal laser

25 scanning microscope will not be able to characterise the development of early erosive
26 lesions and their remineralisation by human saliva in polished human enamel *in vitro*.

27 2.1 Materials and Methods

28 Twenty enamel specimens (5 mm × 3 mm × 2 mm) were prepared from the mid-coronal
29 portion of the buccal and lingual surfaces of extracted caries-free human third molar teeth,
30 using a diamond wafering blade (XL 12205, Benetec Ltd., London, UK). Research ethics
31 approval for use of Human Tissue in this study had been granted (REC number
32 09/H0808/109). From pilot data a sample size calculation revealed that at 5 % level of
33 significance, to test the null hypothesis of correlation between two measures as -0.5
34 against an alternative of -0.76, requires a total sample of 20 samples to achieve the power
35 of 80 % to test the significance of correlation, assuming the bi-variate normal model. The
36 power calculation was carried out using the statistical freeware Gpower (version 3.1.5)
37 [13].

38 The samples were embedded in Protemp4[®] (3M ESPE, Germany) using a dedicated mould
39 former (SyndicadIngenieurbüro, München, Germany) and subjected to a standardised
40 previously published grinding-polishing protocol which resulted in an area of enamel, which
41 was around 5 mm × 3 mm in area with a flatness tolerance of 0.4 µm and homogenous
42 baseline roughness values [14]. All samples were then subjected to an *in vitro* erosion-
43 remineralisation model in order to simulate an early enamel erosion lesion *in vitro*, as
44 described by Young and Tenuata [15]. A 0.3 % citric acid solution was prepared by adding
45 citric acid powder (Sigma-Aldrich, Poole, Dorset, UK) to distilled water, following which the
46 pH was adjusted to 3.2 using a sodium hydroxide buffer and a calibrated pH meter and
47 electrode (WD-35801-00 pH electrode Eutech Instruments, Nijkerk, Netherlands). The
48 solution had a titratable acidity of 19.5 ml, measured as the volume of 0.1 M solution of
49 sodium hydroxide required to raise 20 ml of citric acid solution to pH 7.0 by adding

50 increasing volumes of sodium hydroxide solution followed by agitation and equilibrium for
51 two minutes until the pH reached 7.0.

52 Each sample was immersed in 50 ml of the citric acid solution at room temperature for the
53 following time points: 30 seconds, 1 minute, 2 minutes and 5 minutes, after which the
54 samples were rinsed in distilled water (pH 6.8) and allowed to dry before measurement.

55 Following erosion, the samples were rinsed in distilled water and then immersed in pooled
56 human saliva to allow remineralisation of the eroded enamel lesions. Paraffin-stimulated
57 whole mouth saliva samples were collected from 20 healthy volunteers, following
58 previously published protocols [16]. The collected saliva was ice-chilled and pooled
59 immediately after collection at -80°C for long-term storage. Prior to use, the frozen natural
60 saliva was defrosted in ice time at room temperature $22 \pm 1^\circ\text{C}$. The pH of the saliva was 7.1
61 and the calcium content was 1.4 mmol/l as measured using inductively coupled plasma
62 mass spectroscopy (ICP-MS) [16]. Each 5 samples were immersed in 20 ml of the saliva at
63 room temperature for the following time intervals 1 hour, 6 hours and 12 hours. After each
64 rinsing period the samples were removed from the saliva, rinsed in distilled water and
65 allowed to dry before measurement.

66 The enamel surface microhardness and surface texture was measured at baseline (prior to
67 the erosion / remineralisation model) and again after 30 seconds, 1 minute, 2 minutes and
68 5 minutes of immersion in citric acid (erosion) and after 1 hour, 6 hours, 12 hours of
69 immersion in pooled human saliva (remineralisation). For microhardness, an average
70 Knoop Hardness number (KHN) was calculated from three indentations made using a
71 Duramin-5 Hardness Tester (Struers Inc., Rotherham, UK) with dwell time 5 seconds, load
72 0.981 N and each indentation made 100 μm apart, to ensure that there were no interaction
73 between indents next to each other. During sample repositioning, the live video interface
74 was used to examine the surface to ensure that indents were placed no closer than 100 μm

75 from adjacent indents. The accuracy of the tester was 39.33 KHN as measured using a 600
76 KHN calibrated transfer standard block (Staatliches Materialprüfungsamt Nordrhein-
77 Westfalen, Dortmund, Germany). For surface texture measurement, five 129 $\mu\text{m} \times 129 \mu\text{m}$
78 measurements were made using the x50 objective, 0.95 NA lens of a confocal laser
79 scanning microscope (LEXT OLS4100, Olympus, Tokyo, Japan) employing a 0.2 μm diameter,
80 405 nm wavelength laser beam.

81 In order to select the optimal scale at which to carry out the 3D surface texture analysis for
82 the erosion/remineralisation time points, a correlation analysis between the changes in
83 microhardness and the changes in surface texture at varying relative area-scales was
84 carried out (Sfrax 1.0 <http://www.surfract.com>). This analysis was conducted in order to
85 determine the optimal area scale (in μm^2) at which the surface texture parameters would
86 best highlight the enamel surface features, with reference to the analytical technique
87 microhardness.

88 This area-scale/microhardness correlation data were then used to in order to optimally
89 highlight textural data regarding the relevant features (i.e. the eroded interprismatic
90 enamel pattern) which corresponded to a scale of approximately 20 μm^2 . This information
91 guided the selection of the appropriate filters which were applied to discard unwanted
92 waviness and noise data from the 3D profiles thus ensuring that only data on the relevant
93 feature of interest (i.e. the eroded enamel prisms) was included in the texture analysis. The
94 refinement of the filters was carried out using an iterative process within MountainsMap®
95 whereby representative sample images were taken from each erosion/demineralisation
96 stage and the image analysis workflow was subsequently performed with the 3D data
97 displayed at each of the time points (baseline, during erosion and during remineralisation).
98 In order to confirm the optimal filters, which would highlight pertinent data of the feature
99 of interest in the present study (i.e. the enamel prism structure) most useful low-pass and

100 high-pass filters were determined. As a result of this iterative process, the following filters
101 were applied using MountainsMap[®] surface texture software (Premium v7.1, Digital Surf,
102 France), as shown in Figure 4. Firstly, a 1 μm cut-off robust Gaussian low-pass filter was
103 applied in order to remove high spatial frequencies of the measurement noise. This cut off
104 was chosen to be 1/5th the feature size in that it would not cause any distortion. Following
105 this, a 30 μm cut-off Gaussian high pass filter (i.e. six times the feature size) was applied to
106 remove the irrelevant long wavelength spatial components i.e. waviness. This allowed the
107 mean (SD) roughness parameter S_a to be used to characterise the enamel surface texture
108 at each erosion/ remineralisation timepoint.

109 3.1 Statistical analysis

110 Data were exported to an Excel spread sheet (Microsoft® Office Excel® 2010, Microsoft®
111 Corporation, USA) and statistical analyses performed using GraphPad Prism statistical
112 software (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California
113 USA, www.graphpad.com). Data were initially tested for normality using the D'Agostino-
114 Pearson omnibus test [17]. The data conformed to a normal distribution and therefore
115 means and standard deviations of the groups were reported.

116 For the surface microhardness, the four erosion time points (i.e. 30 seconds, 1 minute, 2
117 minutes and 5 minutes) were compared with baseline values of the sound enamel and for
118 the remineralisation cycles, whereas the three remineralisation time points (i.e. 1 hour, 6
119 hours and 12 hours) were compared with the erosion values of the eroded enamel surface
120 at the maximal erosion time point (i.e. after 5 minutes of erosion) using repeated measures
121 one-way ANOVA, with the Greenhouse-Geisser correction. Tukey's multiple comparisons
122 test was subsequently applied, with individual variances computed for each comparison
123 and $P < 0.05$ considered statistically significant.

124 For the areal scale analysis, the degree to which the microhardness and relative areal scale
125 texture were related was expressed using the coefficient of determination R^2 . This was
126 used to determine the optical scale of filtering to apply to the imaged surfaces prior to
127 calculation of the areal surface texture parameter S_a during the erosion and
128 remineralisation timepoints. Subsequent repeated measures one-way ANOVA was carried
129 out for the S_a data in a similar manner to the microhardness data.

130 4.1 Results

131 Figure 1 shows the mean (SD) enamel surface microhardness (KHN) at baseline, during
132 erosion in citric acid and during remineralisation in pooled human saliva. The mean (SD)
133 Knoop microhardness of the polished enamel samples at baseline (before erosion or
134 remineralisation) was 363 (11) KHN. After erosion in citric acid, there were statistically
135 significant decreases in the microhardness at all the erosion time points in comparison to
136 baseline ($P<0.001$). Each subsequent erosion time point resulted in further statistically
137 significant reductions in the enamel microhardness, such that after 5 minutes of erosion
138 the lowest mean (SD) microhardness value of 266 (10) KHN was reached ($P<0.001$ vs.
139 baseline). Subsequent immersion of the eroded samples in pooled human saliva resulted in
140 a statistically significant increase in the surface microhardness at all the remineralisation
141 times ($P<0.001$ vs. erosion), with the microhardness of the enamel surface demonstrating
142 sequential recovery in hardness over immersion times from a mean (SD) microhardness of
143 316 (13) KHN after 30 minutes remineralisation ($p<0.001$ vs. 5 minutes erosion). The
144 enamel surface showed full recovery to the initial microhardness levels at baseline after 6
145 hours remineralisation 368 (11) KHN ($p<0.001$ vs. 5 minutes erosion).

146 As seen in Figure 2, the hardness vs. area-scale correlation analysis for demineralisation
147 showed that the relative area and the microhardness of the surface was highly correlated
148 over a range of scales between approximately $0.1\ \mu\text{m}^2$ and $20\ \mu\text{m}^2$ ($R^2=0.8$). As seen in
149 Figure 3, for remineralisation the correlation of the area-scale and micro hardness was less
150 highly correlated ($R^2=0.5$) for scales less than $20\ \mu\text{m}^2$. Indeed overall this correlation was
151 weaker than for the demineralisation due to increased variance between results of the
152 remineralisation at the varying time points.

153 Based on this finding that the relevant textural data was lost at scales larger than $20\ \mu\text{m}^2$,
154 the 3D areal surface texture analysis was designed to optimally highlight the relevant

155 features which corresponded to a lateral diameter in the order of 5 μm across. The results
156 of the 3D surface texture analysis following this filtering (removal of unwanted
157 noise/waviness data outside this 5 μm scale) are shown in Figure 5 below. At baseline the
158 mean (SD) S_a of the enamel surface revealed that 8 (2) nm. Immersion of the samples in
159 citric acid for 5 minutes resulted in statistically significant increases in average roughness
160 throughout the erosion time points to reach a mean (SD) S_a of 90 (10) nm after 5 minutes
161 immersion in citric acid ($P < 0.001$).

162 Initial immersion of the eroded samples in pooled human saliva for 1 hour resulted in no
163 statistically significant changes in the average roughness of the surface ($P > 0.05$ vs. 5
164 minutes erosion). However, after all the subsequent remineralisation times there were
165 sequential decreases in the average roughness of the surface over immersion times to
166 finally result in a mean (SD) S_a of 30 (10) nm. This value was statistically significantly
167 reduced in comparison to 5 minutes of erosion ($P < 0.001$) it still remained statistically
168 significantly increased in comparison to the baseline S_a values ($P < 0.001$).

169

170 5.1 Discussion

171 The results of this present study have demonstrated that 3D surface texture analysis of
172 polished enamel samples using confocal laser scanning microscopy is an effective analytical
173 technique for quantitative characterisation of the minute surface changes that occur in
174 human enamel during *in vitro* citric acid erosion ($P < 0.001$ vs. baseline) and *in vitro* human
175 saliva remineralisation ($P < 0.001$ vs. 5 mins erosion). The microhardness analysis
176 demonstrated that the remineralisation in pooled human saliva resulted in a statistically
177 significant recovery of the enamel microhardness at all the immersion times ($P < 0.001$ vs. 5
178 minutes erosion). These sequential increases in microhardness over all the saliva
179 immersion times corresponded with remineralisation re-hardening the surface, such that
180 after 6 hours remineralisation, the enamel surface demonstrated mean (SD) surface
181 microhardness values which were not statistically significant when compared to baseline
182 ($P > 0.05$). This was corroborated by the profilometry data which demonstrated that there
183 was no significant measurable enamel loss either after 5 minutes erosion ($P > 0.05$), nor
184 indeed after 24 hours remineralisation ($P > 0.05$). This confirmed that the *in vitro* erosion
185 model employed in this present study simulated an early enamel erosion lesion with
186 apparently reversible mechanical changes such that the enamel surface could be
187 considered to have been completely remineralised from 6 hours immersion in saliva
188 onwards [15].

189 Area-scale analysis tiles the surface with triangles of constant area and calculates the
190 relative area of the tiling divided by the projected area [10, 18, 19]. By considering triangles
191 of different size, relative area over a range of scales can be calculated. In order to
192 determine the ideal scale at which the most meaningful surface texture data can be
193 examined, the area-scale data was correlated with the microhardness data. For erosion, a
194 good strength of correlation was observed at a range of scales between approximately 0.1

195 μm^2 and $20 \mu\text{m}^2$ ($R^2=0.8$), decreasing above a scale of approximately $20 \mu\text{m}^2$. At scales
196 larger than this, the smaller range textural data was lost (as shown by a sharply decreasing
197 R^2 value), which therefore suggests that the maximum areal feature that an optical surface
198 texture instrument needs to resolve in order to provide meaningful data for enamel texture
199 applications, such as considered in this present one study, is $20 \mu\text{m}^2$, which equates to a
200 lateral resolution in the order of $2.5 \mu\text{m}$. However, superior lateral resolution is desirable as
201 at lateral resolutions inferior to $2.5 \mu\text{m}$, the relevant surface features of the exposed
202 interprismatic pattern seen during early erosion may be severely attenuated. It can
203 therefore be proposed that the optimal range of lateral resolution of a surface texture
204 instrument used to measure polished enamel samples undergoing erosion is less than 2.5
205 μm . At this level of resolution, it is increasingly important to consider the impact of the
206 sample preparation processes as factors such as the depth of enamel removal and the
207 decussation of the enamel prisms at the surface all may possibly influence the magnitude
208 and scale of the surface texture features, both at baseline and as the erosion lesion
209 develops.

210 Historically, a wide variety of surface topography measurement instrumentation has been
211 employed in dental erosion research. Contacting profilometers typically consist of a stylus
212 that physically contacts the surface being measured and a transducer to convert its vertical
213 movement into an electrical signal [20]. Previously the main disadvantage of contacting
214 profilometry was considered to be that the contacting stylus may damage the delicate
215 demineralised surface layer of eroded enamel and thus the effect of the stylus force could
216 have significant influence on the texture measurement results [2, 21]. The stylus diameter
217 also limits the lateral resolution and reflection mode confocal laser scanning microscopy is
218 able to resolve smaller textural features than contacting profilometry [5, 22]. Therefore the
219 results of this present study add weight to the preference for higher resolution optical
220 profilometry for dental erosion assessment [2, 23, 24].

221 The weaker correlation for remineralisation may be explained by the observed increased
222 textural parameters after the first hour of remineralisation. One potential weakness of this
223 study is the pellicle formation may have confounded the measurement of the texture of
224 the enamel surface. The optical instrument used in this present study operated on
225 reflection mode and not subsurface mode [2] and thus the thickness of the acquired
226 enamel pellicle may have interfered with the texture measurement and thus all the
227 remineralisation data. For future studies, a comparison of non-pellicle forming artificial
228 saliva pellicle forming human saliva should be made and indeed use of an alternative
229 texture instrument such as atomic force microscopy may aid the characterisation of the
230 surface, even if a pellicle has been formed [25]. Indeed in this present study, even after 12
231 hours of remineralisation the reduction of the enamel Sa was in steep decline, which could
232 be expected to start to plateau to baseline levels before this point, which again suggests
233 that the remineralisation data be interpreted with caution. This present study, in line with
234 previous studies [4], did not use ultrasonication to remove the pellicle after immersion in
235 artificial saliva, as it takes time for the *in vitro* pellicle to mature which may have adversely
236 affected the rate of remineralisation [26]. Future studies may consider using a non-
237 destructive sub-surface optical instrument to quantify the thickness of the developing
238 salivary pellicle in order to ascertain the relative roles of pellicle formation and
239 remineralisation on the dynamic textural variations seen in this study.

240 The findings of the present study demonstrate the potential for areal surface texture
241 analysis in combination with optical instruments with lateral resolution less than 2.5 μm to
242 characterise the extent of dynamic erosive wear processes occurring at the enamel surface.
243 The observed statistically significant increases and decreases in the enamel surface texture
244 during erosion and remineralisation were highly inversely similar to the changes in
245 microhardness technique, which thus supports the potential for textural analysis
246 characterising the early erosion process *in vitro*. Many authors have previously

recommended a textural characterisation of the erosion process [4, 5], however this present study is the first to have investigated the optimal scale at which to measure textural changes for both erosion and remineralisation. The role of surface texture measurement in erosion has widespread potential to impact on other optical measurement techniques, including reflectometry [3] and optical coherence tomography [27], however the measurement of the micro-texture of a worn curved natural enamel surface presents many more challenges, in terms of both image acquisition and selection of appropriate software which requires understanding of the fundamental nature of the textural changes occurring in enamel erosion as well as the materials and instrumentation required to achieve meaningful data regarding the tribological events that have occurred at the enamel surface [28].

Therefore further research, using appropriate instrumentation and replicating materials, is needed to determine the specific determinants of the most appropriate surface texture parameter for characterisation of the erosive wear process *in vivo* on naturally curved enamel samples. Previous engineering measurement research studies have successfully employed similar areal surface texture analytical techniques to characterise the functionality of the wear processes occurring on difficult-to-access deep drawing dies in the sheet metal industry [29], a finding that has relevance to the clinical situation in which traditional methods for quantification of erosion such as microhardness are not readily applicable.

6.1 Conclusion

In conclusion, high resolution optical surface measurement instrumentation and optimised areal surface texture analytical techniques can effectively characterise enamel demineralisation by citric acid erosion and enamel remineralisation by human saliva in early enamel erosion lesions in polished enamel surfaces *in vitro*...

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Figure 1

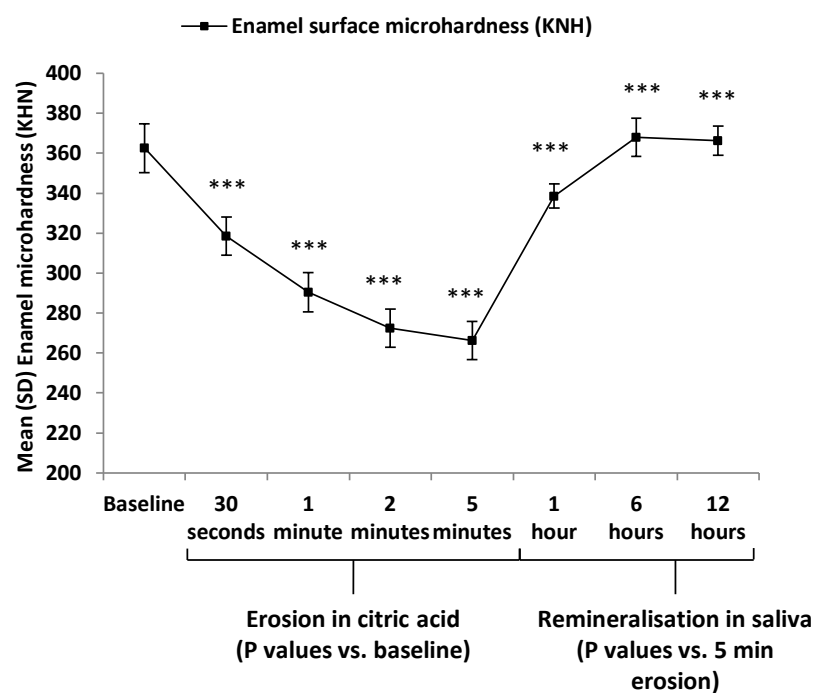


Figure 1 Mean (SD) enamel surface microhardness (KHN) at baseline, during erosion in citric acid (P values vs. baseline hardness) and during remineralisation in pooled human saliva (P values vs. final erosion timepoint ***=P<0.001) (n=20/gp)

Figure 2

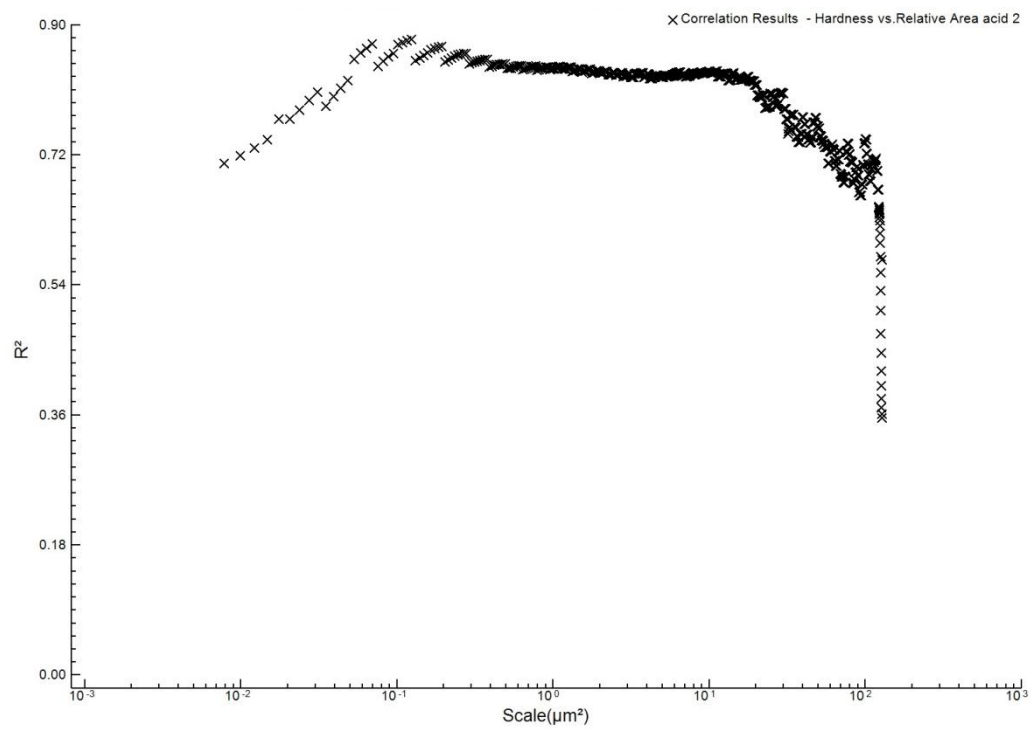


Figure 2 Correlation between the microhardness and the relative area-scale during demineralisation

Figure 3

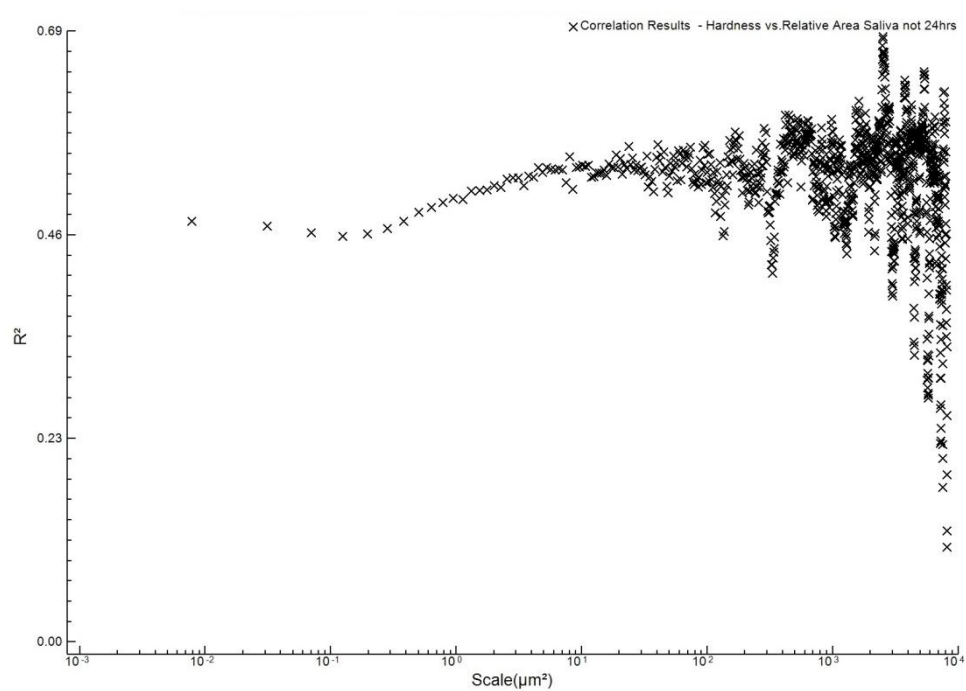


Figure 3 Correlation between the microhardness and the relative area-scale during remineralisation

Figure 4

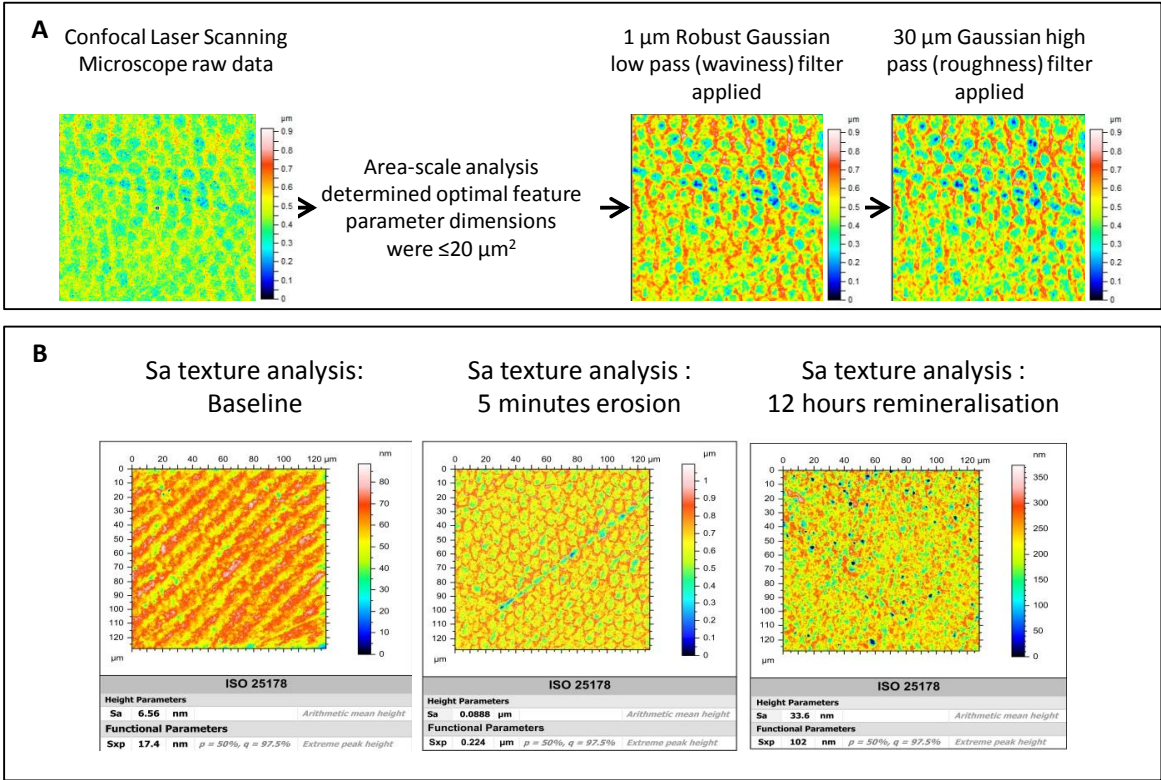


Figure 4 (A) ISO 25178 surface texture image analysis workflow based on results of area-scale analysis correlated with microhardness (B) Representative images of the enamel samples after filtering showing the enamel surface texture at baseline; development of increased surface texture after 5 minutes of erosion and subsequent reduction in surface texture after 12 hours remineralisation **NB. Z-axis scales not uniform**

Figure 5

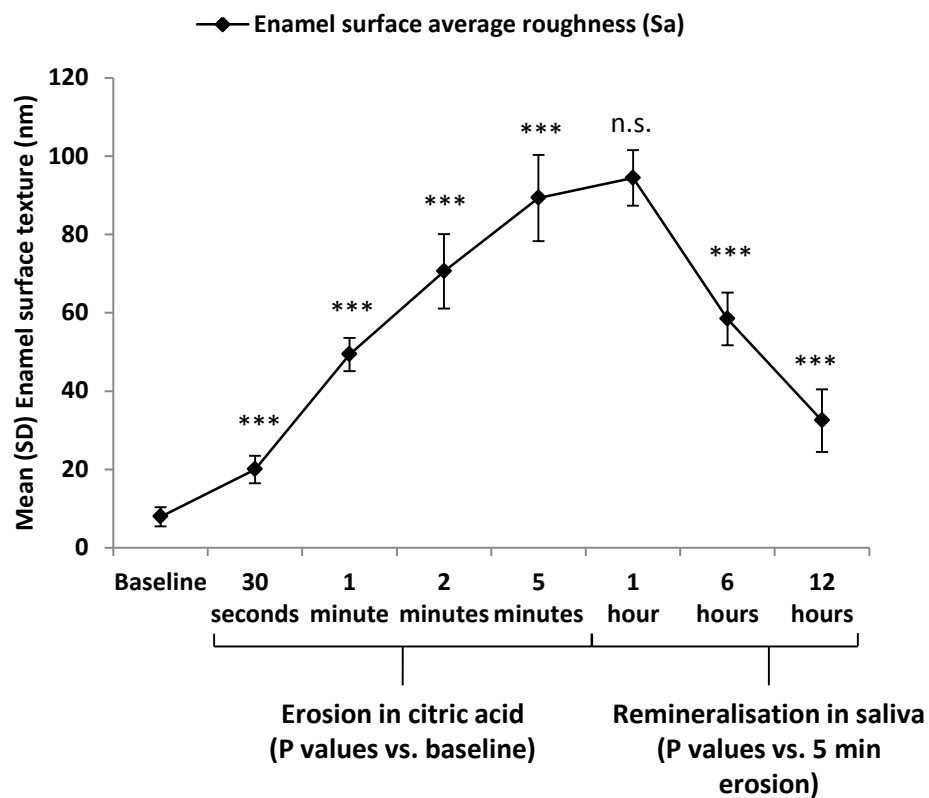


Figure 5 Mean (SD) enamel surface Sa texture (nm) at baseline, during erosion in citric acid (P values vs. baseline hardness) and during remineralisation in pooled human saliva (P values vs. final erosion time point ***= $P < 0.001$ n.s.= $P > 0.05$) (n=20/gp)

Highlights

- We characterise optical and mechanical properties of enamel surfaces during erosion and remineralisation
- 3D imaging of enamel surfaces undergoing erosive wear requires instrumentation with lateral resolution significantly less than 5 μm
- 3D surface texture parameters are able to successfully characterise textural changes in enamel during erosive demineralisation and salivary remineralisation
- The role of surface texture in remineralisation is less clear but suggests that in vivo remineralised lesions remain rougher despite the surface microhardness recovering to baseline levels.